MICROORGANISMS ASSOCIATED WITH FROZEN FISHES (Ice Fish) SOLD AT VARIOUS MARKETS IN ILORIN METROPOLIS

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Abstract

Microorganisms were isolated from the gill, skin, muscle and intestine of 50 samples of fish collected from various markets in Ilorin metropolis. Eleven microorganisms were isolated and identified as; Escherichia coli, Pseudomonas aeruginosa, Proteus spp, Staphylococcus aureus, Micrococcus sp, Salmonella typhi, Shigella spp, Klebsiella pneumoniae, Bacillus subtilis, Aspergillus niger and Aspergillus flavus. The most dominant isolates were Staphylococcus aureus (18.75%) followed by Escherichia coli (16.40%), these together with the highly pathogenic Enterobacteriaceae including Proteus spp, Pseudomonas aeruginosa were also isolated from the samples. Physicochemical analysis such as titratable acidity, fatty acid and moisture content and pH of the samples were determined. Total bacterial and coliform counts were estimated from all parts of the collected samples. The highest bacterial count (cfu/g)1.80±0.36 was recorded at Unilorin market while the least fungal count was at Tipper garage market 1.05±0.52 cfu/g. The negative impacts of the presence of Enterobacteriaceae, Pseudomonas aeruginosa and other microorganisms isolated in fishes were discussed based on their potential pathogenic effect toward human health and their role in enhancing rapid spoilage of fish.

Keywords: Fish, Physicochemical analysis, spoilage and coliform counts

Introduction

Seafood derived from wild fish as well as farmed fish has always been an important source of protein in the human diet. On a global scale, fish and fish products are the most important source of protein and it is estimated that more than 30% of fishes consumed by humans comes from aquaculture (Håstein *et al.,* 2006). According to the Center for Food Safety and Applied Nutrition in Washington (2001), most fish related food borne illnesses are traced to *Salmonella*, *Staphylococcus* sp*, Escherichia coli*, *Vibrio parahemolyticus, Clostridium perfringens, Clostridium botulinum* and *Enteroviruses.* Thampuran *et al.* (2005), have reported that the microbial quality of fish indicated that all tissue samples except muscle were contaminated with faecal coliform where *Escherichia coli* is the most common contaminant and is often encountered in high numbers. According To (Cahill, 1990) the microbiological diversity of fresh fish muscle depends on the fishing grounds and environmental factors around it. Claucas and Ward (1996), suggested that the type of micro-organisms that are found associated with particular fish depends on its habitat. Kvenberg 1991and Rodricks 1991 classified the bacterial pathogens associated with fish as indigenous and non-indigenous. The non-indigenous contaminate the fish or the habitat one way or the other and examples include *Escherichia coli, Clostridium botulinum, Shigella*

dynteriae, Staphylococcus aureus, Listeria monocytogens and Salmonella. The indigenous bacterial pathogens are found naturally living in the fish's habitat for example *Vibrio* species and *Aeromonas* species. The bacteria from fish only become pathogens when fish are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to prevail. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include *Mycobacteium, Streptococcus spp., Vibrio spp., Aeromonas spp., Salmonella spp.* and others (Lippp & Rose 1997).

In Nigeria, there is a large number of public frozen seafood processing services distributed along the country, where a considerable number of people buy their frozen seafood product daily. Serious consequences relating to national productivity and development can arise from lack of hygiene and sanitation in such outlets. There have been several reports on the health risks associated with the consumption of processed seafood, ranging from allergic reactions, stomach and intestinal cancerous growths, a general degeneration of peripheral cellular tissues, to gradual breakdown of the digestive and excretive systems in a statistically high percentage of people examined. Few of these reports however, have looked at the likely risks from a microbiological food safety point of view (Edema *et al.,* 2005). According to Higgins (2007) anyone who works in food safety sooner or later discovers that one of the most valuable tools for prevention is simply reading about and understanding how past outbreaks have occurred. Using major and frequently famous or at least news worthy outbreaks, Phyllis (2007) illustrates how critical factors come together to produce tragic and largely preventable results.

The microbiological safety of food is achieved by as far as possible ensuring the absence of pathogenic microorganisms and by all means preventing their multiplication (Edema & Omemu, 2004). The Hazard Analysis Critical Control Point (HACCP) concept is used to identify microbiological vulnerable points in the food production process and processing, to determine the most appropriate methods of control to be applied, usually such methods as improved handling techniques, monitoring of temperature and more intensive supervision (Edema & Omemu, 2004).

The bacterial flora on newly-caught fish depends on the environment in which it is caught rather than on the fish species (Shewan, 1977). Fish caught in very cold, clean waters carry the lower numbers whereas fish caught in warm waters have slightly higher counts. Very high numbers of microorganisms are found on fish from polluted warm waters. Many different bacterial species can be found on the fish surfaces. The bacteria on temperate water fish are all classified according to their growth temperature range as either psychrotrophs or psychrophiles.

Psychrotrophs (cold-tolerant) are bacteria capable of growth at 0°C but with optimum around 25°C. Psychrophiles (cold-loving) are bacteria with maximum growth temperature around 20°C and optimum temperature at 15°C (Morita, 1975). In warmer waters, higher numbers of mesophiles can be isolated.

However, there are lots of factors that influence the survival rate of bacteria in food especially animal products. The chief compositional factors of the fish that influence microbial activities are temperature, hydrogen ion concentration, moisture, oxidation reduction potential, nutrient and the presence of inhibitory substances or barriers. The nutrient content of a food, their kinds and proportion are all other factors that determine the survival and growth rate of organism in food, added purposely or accidentally or developed there by growth of microorganisms (Okonko *et al.,* 2008).

The objective of this study is to isolate and identify microorganisms that are associated with frozen fishes.

Materials and Methods

Sample Collection: Frozen fish samples were obtained from 5 different markets within Ilorin metropolis. Sampling was done using sterile nylon bags. The bags were labeled appropriately and placed in an ice-chest and taken to the laboratory immediately where microbiological analyses of all the samples were done.

Physicochemical Analysis

Determination of pH of the samples: Five gram of each sample was weighed and ground with sterile mortar and pestle. It was transferred into beakers, 40ml of distilled water was added into each beaker. The mixture was allowed to settle for 10minutes after which the glass electrode of the pH meter was dipped into the solution. The pH reading for each sample was done 3 times and recorded (Lunette *et al.,* 1985).

Determination of Moisture Content: Five grams of each sample of frozen fish was weighed into a known weight of oven dried crucible. The samples were then carefully arranged in an oven after which the oven was switched on and set at temperature of 80°C. The samples were weighed at intervals until a constant weight was obtained. The differences in the weight was considered as the moisture content of the sample and it was expressed as a percentage using the formula below:

$$\%\text{moisture content} = \frac{X-Y}{X} \times 100$$

Where X = initial weight of the sample i.e. 5g

Y= final weight of the sample (AODAC, 1980).

Determination of Titratable Acidity: Five grams of each grounded samples of frozen fish was mixed with 50ml of distilled water. The mixture was filtered using a filter paper. 20ml of the filtrate was pipette into a conical flask, 2drops of phenolphthalein was added as indicator and it was titrated against 0.1M sodium hydroxide. The volume of sodium hydroxide used was noted. This was replicated for each sample and the mean results were recorded (Kirk *et al.,* 1971).

Fatty Acid Determination: Four grams of each frozen fish samples were grounded using sterile mortar and pestle. The grounded samples were mixed with 5ml of warm alcohol and 1ml of phenolphthalein was added. The mixture was then titrated against 0.1m sodium hydroxide. The formula for the calculation goes thus.

$$\text{Fatty acid} = \frac{V \times N \times M}{10 \times W}$$

Where V = Volume of sodium hydroxide used
N = Number of moles of sodium hydroxide (0.1)
M = Molecular weight of fatty acid (282)
W = Weight of sample (4g) (Nielsen, 1998).

Sugar Determination: To determine the sugar content of frozen fish, 2g of each sample was grounded using sterile mortar and pestle. The grounded samples were mixed with 20ml distilled water.

The mixture was filtered and 1ml of the filtrate was then transferred into a beaker by means of a pipette. One milliliter of 5% phenol solution of concentrated H_2SO_4 was subsequently added to the content of the beaker.

The samples were placed on the shaker and were shaken for 20minutes. The optical density was then taken using a thermo specific Genesys at a wavelength of 490nm. The readings were recorded. The optical density of glucose was taken as the standard.

The sugar content of the sample was determined on a graph of the optical density of sugar plotted against its concentration in mg/g (AOAC, 1980).

Determination of Ash Content: Two grams of each frozen fish sample was weighed into a sterile crucible of a know weight and then placed in a furnace which was set at 60^0C for 5hours and the furnace left to cool. The samples were brought out by means of a forcep. The weights of the crucibles containing the samples were determined using a weighing balance and the percentage ash content was calculated thus:

$$\% \text{ Ash content} = \frac{\text{Weight of ash} \times 100}{\text{Original weight of sample}}$$

(AOAC, 1980).

Protein Content Determination: Using the Kjedhal method, two grams of the ground fish samples was mixed with five grams of Sodium sulphate, one gram of Copper sulphate and 20ml of concentrated tetraoxosulphate (VI) acid was then added. The flask was then carefully heated first at low temperature for about 30minutes, then increased until it boiled and then allowed to digest. It was allowed to cool and then diluted with 250ml distilled water. 5ml of the resulting solution was then transferred into conical flask and excess concentration of sodium hydroxide was added to make a strong alkaline solution so that all ammonical nitrogen changed to ammonical sulphate. Two percent boric acid was then added. It was then titrated against 0.01M hydrochloric acid. The resulting color was blue.

$$\text{Total nitrogen (g/100cm}^3) = \frac{14 \times 100 \times \text{volume of acid}}{1000}$$

$$\text{Protein g/100cm}^3 = \frac{14 \times 0.01 \times a \times b \times 6.25 \times 100}{c \times d}$$

Where:
a = volume of acid used
b = volume of distillate (250ml)
c = volume of distillate used for titration (5ml)
d = weight of sample (2g) (Kirk *et al.,* 1991).

Bacteriological Analysis

Enumeration of Total Bacteria: For enumeration of bacteria, the internal flesh of the frozen fish samples was used. One gram of the internal flesh of each of the fish samples were weighed and grounded with sterile mortar and pestle. It was then suspended in 10ml of sterile distilled water to make the stock solution One milliliter of the stock was added to 9ml of sterile distilled water to make 10^{-1} dilution. This same procedure was repeated until 10^{-4} dilution was obtained. Inocula for total bacteria and coliform count were taken from 10^{-4} dilution and then transferred to the center of the base of a sterile petridish after which sterile nutrient agar was added aseptically.

Isolation of Bacteria: One gram of the fish samples were weighed and mercerated with sterile mortar and pestle. It was then suspended in 10ml of sterile distilled water aseptically to make the stock. For isolation of bacteria, direct streaking method was used. The plate that were used for direct streaking were dried in the oven for some minutes. Inoculating loop was flamed to redness and then allowed to cool after which it was used to pick loopful of the suspended fish sample and then used to streak the surface of the plate. This was done under aseptical conditions to avoid contamination.

Fungi Analysis

Enumeration of total fungi: For enumeration of fungi, the internal flesh of the frozen fish samples was used. One gram of the internal flesh of each of the fish samples were weighed and grounded with sterile mortar and pestle. It was then suspended in 10ml of sterile distilled water to make stock and I ml was added to 9ml of sterile distilled water to make 10^{-1}dilution. Inoculum was taken from 10^{-2} dilution and then cultured on Sabouraud dextrose agar and incubated at room temperature for 48- 72hours.

Results

Table 1: Physicochemical characteristic of the samples

Sample code	Crude protein (%)	Crude fat (mol/ml)	Total ash (%)	pH	Moisture content (%)	Titratable acidity (ml)	Sugar content (mg/g)
A	75.281±20.13	1.20±0.04	9.80±1.94	6.70±0.19	6.42±1.04	1.75±0.25	1.53±0.41
B	86.10±13.45	1.21±0.04	8.90±0.91	6.71±0.17	6.63±1.12	1.75±0.27	1.39±0.30
C	95.27±12.17	1.23±0.05	9.69±1.03	6.61±0.30	6.17±0.85	1.60±0.21	1.43±0.14
D	89.10±15.86	1.25±0.04	9.70±0.95	6.68±0.17	6.71±1.11	1.80±0.23	1.51±0.35
E	82.72±18.93	1.20±0.06	9.40±1.01	6.62±0.19	6.71±1.13	1.78±0.25	1.46±0.26

Key: A = Unilorin market, B = Tipper garage market, C = Yoruba road market, D = Oja tuntun market, E = Ipata market n = 10.

Table 2: Total Bacterial, coliform and fungal count

Sample code	Bacterial count (cfu/g)× 10^4	Coliform (cfu/g)× 10^4	Fungal count (cfu/g)× 10^2
A	1.80 ± 0.36	1.10 ± 0.18	1.20 ± 0.56
B	1.50 ± 0.32	1.20 ± 0.21	1.05 ± 0.52
C	1.60 ± 0.43	1.11 ± 0.20	1.11 ± 0.63
D	1.40 ± 0.39	1.10 ± 0.15	1.38 ± 0.49
E	1.60 ± 0.49	1.13 ± 0.25	1.25 ± 0.59

A = Unilorin market
B = Tipper garage market
C = Yoruba road market
D = Oja tuntun market
E = Ipata market
Each value represents the mean of 10 replicates.
n = 10.

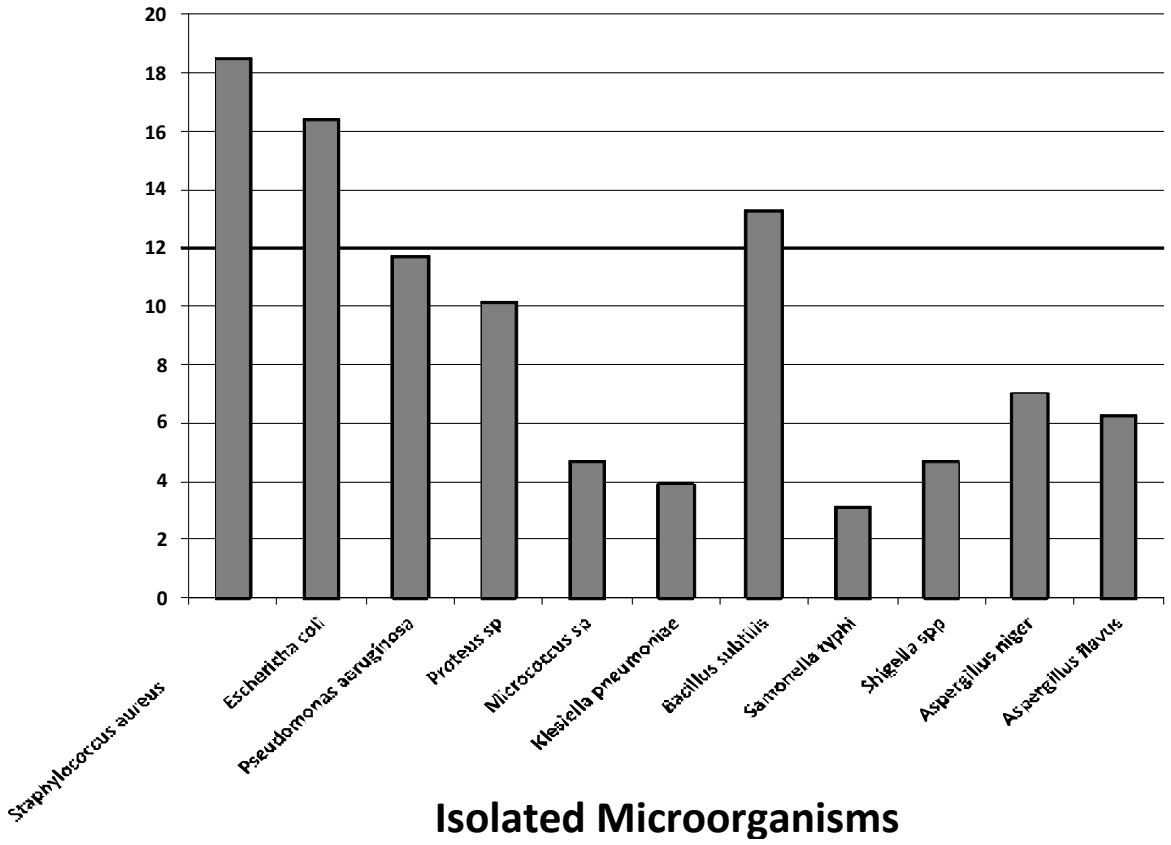

Fig. 1: Frequency of occurrence of isolates (%)

Discussion

Fish is an important food commodity in the international trade but they deteriorate rapidly especially when storage facilities are lacking. It has been widely accepted as a good source of protein and other elements necessary for the maintenance of healthy body. Frozen fish were examined for the presence of microorganisms. The total bacterial count ranged from $1.40 \pm 0.39 \times 10^4$ cfu/g - $1.80 \pm 0.36 \times 10^4$ cfu/g. These values are within the permissible range of ice fish product. The total coliform count also ranged from $1.10 \pm 0.15 \times 10^4$ cfu/g - $1.20 \pm 0.21 \times 10^4$ cfu/g, while the total fungal count ranged from $1.05 \pm 0.52 \times 10^2$ cfu/g - $1.38 \pm 0.49 \times 10^2$ cfu/g. From the result of this study, it can be seen that frozen fish sold in the markets has high contamination which may be as a result of certain factors like temperature which favours some organisms growth and lack of personal hygiene by the handlers and fishes taken in contaminated water which may contain faecal matter in their ecosystem.

The bacteria isolated and identified from the gill, skin, muscle and intestine of different processed frozen fishes were: *Escherichia coli, Pseudomonas aeruginosa, Proteus sp, Staphylococcus aureus, Micrococcus sp, Klebsiella pneumoniae, Salmonella typhi, Shigella spp and Bacillus subtilis.*

Most of the bacterial isolates were of the family *Enterobacteriaceae.* Faeces and untreated water are the most likely source of these contaminants. The presence of indicator organisms such as *Escherichia coli* might be the result of possible contamination during sales or

unhygienic handling of fish right from the processing plants. Thampuran *et al*. (2005) reported that the microbial quality of the tilapia indicated that all tissue samples except muscle tissues were contaminated with fecal coliform where *Escherichia coli* is the most common contaminant and is often encountered in high numbers. The presence of *E. coli* as well as verotoxigenic *E. coli* O157:H7 in fish meal was also investigated by some other authors (Ayulo *et al*., 1994; Hwang *et al.,* 2004; Thampuran *et al*., 2005; Ristori *et al.*, 2007). The presence of these organisms could be an indicator for faecal contamination of water used in rearing the fishes and this might have adverse effect on the health of the consumers (Okonko *et al.,* 2008). *Escherichia coli* are the most predominant enteric bacteria found. Human beings are believed to be carriers of toxigenic and invasive strain of *Escherichia coli* isolated from foods (Nielson *et al.,* 1998). *E.coli* has been implicated as pathogens of men causing different types of gastro intestinal infection.

Bacillus subtilis are spore formers and are known to produce toxins. The presence of this organism in frozen fishes suggest that fish sample might have been contaminated by dust carrying the resistant forms of the organism because a large number of *Bacillus sp* often occur in the soil and dust, such that any slight disturbance of the dust can lift up their resistant forms into the air. *Bacillus subtilis* has been attributed to cause two distinct syndromes: The “diarrheal syndrome” which occurs between 12 – 24 hours after ingestion of proteinous food. The symptom includes; abdominal pain, diarrhea and nausea while the “Emetic syndrome” which causes illness is characterized by rapid onset of nausea, vomiting and malaise (Chichester *et al.,* 1973).

The presence of *Staphylococcus aureus* in the sampled fishes, is of human health concern. Their significant presence in these frozen fishes might be due to contamination from the source as a result of handling by processors. This is in accordance with the previous study by some authors in Nigeria and outside Nigeria (Okonko *et al*., 2008, 2009). Food poisoning by the enterotoxin produced by *Staphylococcus aureus* could result from consumption of frozen fish with *Staphylococcal* toxin. Improper handling and poor hygiene might lead to the contamination of frozen fish and this eventually affects the health of the consumers (Dubey *et al.,* 2004).

The isolation of *Pseudomonas* sp. from the fish samples is significant because this bacterium plays a considerable role as potential human pathogenic bacteria for human and as an indicator of food quality especially as spoilage organism. This is in consonance with what was previously reported by Koutsoumanis and Nychas (2000), Jeyasekaran *et al*. (2006) and Yagoub (2009) that identified *pseudomonads* as a good spoilage index. *Pseudomonas aeruginosa* could cause septicemia in person already in poor health (Baker & Silverton, 1978).

The Isolation of some highly pathogenic agents such as *Salmonella typhi* and *Shigella* sp. and potential pathogenic organisms such as *Klebsiella pneumoniae and Proteus* sp. indicated faecal and environmental pollution and these supported the findings of Yagoub *et al.* (2004) who isolated pathogenic and potential pathogenic organisms from tap water that originated from Nileriver. This also confirms the findings of Koutsoumanis and Nychas (2000);

Gonzalez-Podriguez *et al.* (2001) and Herrera *et al.* (2006) who isolated similar organisms from fish and fish products.

The total fungal counts ranged from $1.05\pm 0.52 \times 10^2$ cfu/g – $1.38\pm 0.49 \times 10^2$ cfu/g.The fungi isolated were *Aspergillus niger and Aspergillus flavus.* These fungi are found to be associated with the contamination of frozen fish and they are not indigenous microorganisms of fish i.e. they are not part of the fish from its habitat. Rather, they are opportunistic (contaminants) microorganisms which find their way into the fish when the condition or environment on or within the fish is favorable for their growth

The percentage moisture within the fish which is as low as 4%, allows molds to thrive well in it. The presence of the fungi might be accidental but they make use of this opportunity to aggrevate the contamination of the fish because the environment is conducive for their survival (Essien *et al.,* 2005). The association of some of these fungal species with fish has earlier been reported (Fafioye *et al.,* 2001).

The occurrence of *Aspergillus* species is not surprising because they are fast growers. The consumption of fish that is contaminated by any of *Aspergillus* species is dangerous to human health because they produce aflatoxin (WHO, 2002). *Aspergillus* species causes Aspergillosis; also the aflatoxin produced by it is carcinogenic and can cause respiratory infections especially in immune-suppressed individual. It can also cause hypersensitivity or allergic reaction and acute liver cirrhosis (Willey *et al.,* 2008).

The pH and the moisture content of the fish influence the organisms which can survive in it and may also determine whether the contaminating microorganisms could produce toxin. From the result, pH ranged between 6.61 – 6.70 which provides a suitable environment for the growth of bacteria. The hindering factor however, is the temperature of storage of sample which is very low and can only allow the growth of specific bacteria i.e. the psychrophiles ($\leq 20^0$c) (Petegem *et al .,* 2002).

Conclusion

Fish are preserved in a frozen state in order to lengthen the shelf life. From this study, it is therefore suggested that frozen fishes processing operators should be educated on the adverse effect of using untreated or polluted water for processing as these could serve as source of faecal contamination. However, the retailers should observe strict hygiene measures so that they will not serve as source of chance inoculation of microorganisms and contamination of these processed frozen fishes.

When frozen fishes are consumed without proper cooking, there is the likelihood of endangering the health of the consumers especially when the organism present includes pathogenic ones.

Recommendation

Finally, it is also preferable that these processed frozen fishes are properly washed with very clean water and should be properly cooked before dressing for consumption so as to get rid

of all microorganisms that might have colonized it and to inactivate all spores including toxins already produced in it if there is any. In no situation should the frozen fishes be consumed without any form of pre-treatment because they might serve as source of infection to the consumers.

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